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(54) Title: FORMATION OF CONJUGATED UNSATURATED FATTY ACIDS

(57) Abstract

The present invention relates to the use of certain bacterial strains found among food grade bacteria, particularly among dairy starter cultures, to produce conjugated linoleic acid (CLA) *in vitro* by fermentation. In particular strains of propioni- or bifidobacteria, such as Propionibacterium freudenreichii or Bifidobacterium breve, are used. Said bacteria may be used to provide food or feed products enriched in CLA, and also pharmaceutical products containing CLA as active ingredients.

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FORMATION OF CONJUGATED UNSATURATED FATTY ACIDS

FIELD OF THE INVENTION

The present invention relates to the use of certain bacterial strains found among food grade bacteria, particularly among dairy starter cultures, to produce conjugated linoleic acid (CLA) *in vitro* by fermentation. Said bacteria may be used to provide food or feed products enriched in CLA, and also pharmaceutical products containing CLA as active ingredient.

BACKGROUND OF THE INVENTION

Conjugated linoleic acid (CLA), a mixture of isomers of octadecadienoic acids with conjugated double bonds, has attracted considerable attention because of its potentially beneficial biological effects. Pariza and co-workers reported initially that CLA inhibits the initiation of mouse skin carcinogenesis (Ha *et al.* 1987; Pariza and Hagraves 1985). CLA is also involved in the inhibition of mouse forestomach and rat mammary tumorigenesis (Ha *et al.* 1990; Ip *et al.* 1991). Shultz *et al.* (1992) found that physiological concentrations of CLA are cytostatic and cytotoxic to human malignant melanoma, colorectal and breast cancer cells *in vitro*. More recently, CLA was reported to be effective in preventing the catabolic effects of immune stimulation (Cook *et al.* 1993; Miller *et al.* 1994). Of the individual isomers of CLA, cis-9,trans-11-octadecadienoic acid (c9,t11-18:2) has been implicated as the most important isomer in terms of biological activity because apparently only this isomer is incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers (Ha *et al.* 1990; Ip *et al.* 1991).

As shown above, CLA seems to have beneficial biological effects in certain animal models. Further, preliminary results indicate that CLA possibly may reduce the blood lipid levels, reduce the atherosclerosis effect and beneficially influence the body composition of experimental animals.

Dietary sources of CLA are mainly milk and meat of ruminant origin. Dairy products are one of the major dietary sources of CLA, of which c9,t11-18:2 is the major isomer (Chin *et al.* 1992; Fogerty *et al.* 1988) and represents more than 80% of the total CLA.

CLA is produced from polyunsaturated fat by certain rumen micro-organisms (Shorland 1955). Kepler and Tove (1967) showed that the first intermediate in the biohydrogenation of linoleic acid by rumen bacteria *Butyrivibrio*

fibrisolvens is c9,t11-18:2. More rercently, it was reported that the intestinal flora in rats is also capable of converting free linoleic acid to cis- and trans-9,11-octadecadienoic acid (c9,t11-/t9,c11-18:2) (Chin *et al.* 1994).

Further studies on the production of CLA by bacteria have been carried out (Fairbank *et al.* 1989; Jack *et al.* 1994). The strains in these references are not identified but are pathogenic bacteria.

The amount of CLA necessary to provide a physiological effect in humans may be calculated based upon the knowledge of the effect of ω-3 fatty acids, particularly EPA and DHA, and based upon results from animal tests. Thus, up to 3 g of CLA per day, say 1-3 g per day in the human diet seem to be necessary to achieve an impact on the metabolism in humans.

The normal human intake of CLA from the diet of today corresponds to about 0.1 to 0.5 g of CLA per day.

As stated above, dairy products are one of the major dietary sources of CLA, in particular milk. The content of CLA in milk fat varies largely and is normally about 0.5% (5 mg/g fat) in commercial milk produced in Sweden. In feeding experiments we have noted levels of 1.5% with individual cows. It seems to be possible to increase the CLA contents of the milk fat up to 2% by feeding the cows with a combination of feed enriched in linoleic acid and coarse fodder.

It has been proposed that cheese contains higher levels of CLA than other products and that the CLA contents in dairy products may be increased by processing and storage (Ha *et al.* 1989; Shantha *et al.* 1995). However, the increase of the concentration of CLA in dairy products resulting from processing is small compared to the large variation in unprocessed milk.

It has also been reported that feed efficiency and growth rate markedly improved when several animal species were fed with CLA (Cook 1995). Rats, mice, chickens and rabbits fed with CLA in their diets ate less overall, indicating improved feed efficiency. Their body fat dropped but body protein increased. This also indicates the usefulness of CLA as an animal feed additive or food ingredient to achieve further beneficial health effects.

From the above it will clearly appear that the amount of CLA in a regular diet cannot reach levels from which human can benefit. For instance, if the required amount of CLA is solely from milk consumption, this would necessitate a consumption of 25 liters/day (the normal milk containing about 4-5 mg of CLA per gram fat). The only way to go is to produce foods enriched in CLA and this should be done without compromising the intake of fat and cholesterol. Thus,

there is an obvious and eager need for a method of producing food and feed products enriched in CLA and this need is satisfied by the present invention.

OBJECT OF INVENTION

In view of the above, the main object of the present invention is to provide methods and means, without compromising the intake of fat and cholesterol, for producing food and feed products enriched in CLA, the major isomer of which is c9,t11-18:2. Another object is to produce an additive enriched in CLA, the major isomer of which is c9,t11-18:2. Other objects of the invention are evident to the skilled man from the description and claims to follow.

SUMMARY OF THE INVENTION

We have surprisingly found that certain bacterial strains found among food grade bacteria, particularly among dairy starter cultures, have the capacity to produce CLA *in vitro* having essentially the same isomer composition as that of milk. Thus, according to one embodiment the present invention relates to the use of such food grade bacteria to produce CLA and particularly the use of such food grade bacteria for the preparation of CLA-enriched food or feed products by fermentation of said food grade bacteria.

A second embodiment of the invention relates to a food or feed product enriched in CLA either by fermentation with said CLA-producing food grade bacteria or by the addition of a food or feed grade additive containing CLA prepared by fermentation with said CLA-producing strains.

A third embodiment of the invention relates to a process for the preparation of the food or feed product according to the second embodiment by fermenting a product to be enriched in CLA, under CLA-producing conditions, with said CLA-producing food grade bacteria.

A fourth embodiment of the invention relates to a further process for the preparation of the food or feed product according to the second embodiment by adding to a product to be enriched in CLA a food or feed grade additive containing CLA prepared by fermentation with said CLA-porducing strains.

A fifth embodiment of the invention relates to the additive used for the process of preparation according to the fourth embodiment and being a CLA-enriched product prepared by fermentation with said CLA-producing food grade

bacteria.

DESCRIPTION OF THE DRAWINGS

FIG. 1A and B. GC chromatogram of the fatty acid composition of the media (A) and the bacterial pellet (B), efter the growth of *Propionibacterium freudenreichii* subsp. *freudenreichii* Propioni-6 (PFF6) in MRS-broth containing 25 $\mu g\ ml^{-1}$ of free linoleic acid. Anaerobic incubation at 20°C for 72 h. The numbered peaks represent the following fatty acid: 1) linoleic acid; 2) cis9,trans11/-trans9,cis11-18:2; 3) trans10,cis12-18:2; 4) trans9,trans11/trans10,trans12-18:2; 5) internal standard.

FIG. 2A and B. Formation of conjugated linoleic acid by propionibacteria in MRS-broth supplemented with different amounts of free linoleic acid. Anaerobic incubation at 20°C for 72 h. A) cis9,trans11/trans9,cis11-18:2; B) trans9,trans11/trans10,trans12-18:2; ■, *Propionobacterium freudenreichii* subsp. *freudenreichii* ATCC 6207 (PFF); ●, *P. freudenreichii* subsp. *shermanii* 9093 (PFS); Δ, *P. freudenreichii* subsp. *freudenreichii* Propioni-6 (PFF6).

FIG. 3A and B. Effects of different concentrations of free linoleic acid on the growth of propionibacteria in MRS-broth. Anaerobic incubation at 20°C for 72 h. A) final viable counts; B) differences between the final and the initial pH. ■, *Propionibacterium freudenreichii* subsp. *freudenreichii* ATCC 6207 (PFF); ●, *P. freudenreichii* subsp. *shermanii* 9093 (PFS); ▲, *P. freudenreichii* subsp. *freudenreichii* Propioni-6 (PFF6).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the surprising discovery that certain bacterial strains found among food grade, i.e. non-pathogenic, bacteria, particularly among dairy starter cultures, have the ability of converting linoleic acid to CLA *in vitro*, the major isomer of which is c9,t11-18:2.

Thus, according to one embodiment, the invention relates to the use of bacterial strains having the ability of producing CLA *in vitro* by converting linoleic acid to CLA upon incubation, the bacterial strains being found among food grade (non-pathogenic) bacteria. Said bacteria are used for the production of food or feed products enriched in CLA or pharmaceutical products containing CLA as active ingredient.

Any food grade bacterial strain having the capacity of forming CLA *in vitro*

by converting linoleic acid to CLA may be used, such as strains of lactobacilli, lactococci, streptococci, propionibacteria and bifidobacteria, particularly strains used as dairy starter cultures. In order to establish whether a particular food grade bacterial strain is useful for the purpose of the invention, the strain in question can be tested by the skilled man for its ability to produce CLA from free linoleic acid *in vitro*, according to the test procedure described below under "MATERIAL AND METHODS".

In the following, the claimed invention will be described and discussed with particular reference to dairy starter cultures as CLA-producing food bacteria but it is to be understood that the invention is not in any way restricted to said starter cultures. Further, the claimed invention will be described and discussed with particular reference to dairy products and especially milk as food or feed product but it is to be understood that the invention in no way is restricted to said dairy products.

As indicated above, under "SUMMARY OF THE INVENTION", we have found that certain strains of food grade bacteria are able to produce CLA *in vitro*, viz. all strains of the food grade bacteria cannot produce CLA. For instance, we studied six strains of propionibacteria, three of them can form CLA; we studied three strains of bifidobacteria, only one of them can form CLA. Thus, we have found that the CLA formation is strain-dependent which is quite surprisingly and in contrast to the results of Jack *et al.* 1994.

There are several published papers having studied the CLA concentration in different products, including Jiang *et al.* 1997 (in press) discussing CLA in Swedish dairy products, and no increase of CLA was found in different fermented dairy products cultured by different bacteria. Therefore, the production of CLA is not a common behaviour of dairy starter cultures.

We have found three dairy starter culture strains being particularly useful and being found to convert free linoleic acid to extracellular CLA, viz. two strains of *Propionibacterium freudenreichii* (*P. f.*) subsp. *freudenreichii* (ATCC 6207 and Propioni-6) and one strain of *P. f.* subsp. *sheramnii* (9093). The highest level of CLA formed in the fermentation broth was 265 $\mu g\ ml^{-1}$. Of the different isomers, cis- and trans-9,11-octadecadienoic acid represented more than 70% of the total CLA formed.

The CLA-producing strains of the invention are used for the preparation of food or feed products enriched in CLA. By "enriched in CLA" is meant, throughout this specification and claims, that the product in question contains

CLA in a concentration above what is found in the food or feed product before the enrichment and that has an impact on the metabolism to provide the desired physiological effect.

Thus, according to a second embodiment, the invention relates to a food or feed product enriched in CLA, the CLA-enrichment having been achieved by incubation of the product with said CLA-producing strains or by the addition to the product of a food or feed grade additive containing CLA prepared by incubation with said CLA-producing strains.

By "food product" is meant, throughout the present description and claims, a dietary product intended for human consumption and examples are ordinary foods, funktional foods, parnut foods and medicinal foods. By "feed product" is meant a product intended for animal consumption, such as a feed additive.

The term "pharmaceutical product" is meant, throughout the present description and claims, to include such products as medicinal products, drugs, natural remedies, OTC products etc.

The food product of the invention may be a dairy product such as milk, fermented milk, cheese, yellow fats such as butter and dairy spreads, ice-cream, or imitation dairy products containing vegetable fat or proteins, or fermented foods of plant and animal origin. When incubating milk (3% fat content) with a CLA-producing propionibacterium strain, up to 20 mg of CLA per gram of milk fat may be formed from 1 g of linoleic acid. This corresponds to an enrichment of about 0.5 g of CLA per liter of milk (3% fat content). Higher levels of CLA can be expected to be formed when using optimal conditions and strains. A further feature is that the CLA produced in milk according to the invention shows the same isomeric pattern as the CLA naturally contained in the milk.

The CLA-enriched milk fermented according to the invention may be concentrated, or processed to other CLA-enriched dairy products as is well known to the man skilled in the art. Such dairy products are hard and fresh cheeses, dairy spreads, special fermented milk products, processed cheese etc. or can be milk powders or similar products with fat.

According to a third embodiment, the invention relates to a process for the preparation of the CLA-enriched food of feed product of the invention, the process being characterized in that the product to be enriched in CLA is fermented or incubated with a CLA-producing bacterial strain according to the invention, using linoleic acid as substrate, under CLA-producing conditions. Said conditions can easily be established by routine experiments. Thus, in case of preparing CLA-

enriched milk using a propionibacterium strain, linoleic acid and the strain in question are added to milk which is then incubated at about 22°C for up to 72 hours. An enriched level of CLA of 0,5 g/litre is normally reached after 24 hours incubation. Emulgators may be added to promote the formation of CLA and examples of such emulgators are Tween 80 and lecithins.

An advantage of the process of the invention is that the fermented food product, i.e. milk, can be used as such for consumption without any need of separation or purification steps.

The CLA-enriched food or feed product can be prepared by using a vegetable oil containing linoleic acid in the triglyceride form, such as corn oil. This can be hydrolysed by lipase to obtain free linoleic acid. The lipase can be added as such or formed by the microbial strain used. Thus, the lipase-treated oil can be used in food or feed products, such as dairy products, and the free linoleic acid can be converted to CLA by the CLA-producing bacteria of the invention.

A fourth embodiment of the invention relates to a further process for the preparation of the CLA-enriched food or feed product, the process being characterized in that a food or feed grade additive is added to the product to be enriched in CLA, the additive in turn having been prepared by fermentation or incubation with a CLA-producing bacterial strain of the invention. This is particularly useful when preparing animal feed products where a feed additive containing increased CLA level is added to the animal feed.

A fifth embodiment of the invention relates to the above additive used for the preparation of the CLA-enriched food or feed products of the invention and being as such a CLA-enriched product prepared by fermentation or incubation with a CLA-producing bacterial strain according to the invention. The additive may be CLA as such or CLA in concentrated form such as concentrated fermentation broth containing CLA.

The additive is produced using food grade bacteria by ordinary fermentation under anaerobic conditions using a substrate, pH and temperature allowing said food grade bacteria to grow and produce CLA. The fermentation process can use known conditions to increase yield, such as growth at constant pH and continuous or semi-continuous addition of linoleic acid, with or without removal of CLA from the medium. By using food-grade bacteria and ingredients, an additive suitable for food and feed products can be achieved.

MATERIALS AND METHODS

Strains

Seven strains of lactobacilli, four strains of lactococci, two strains of streptococci and six strains of propionibacteria were included in the study (Table 1). The strains were obtained from the National Collecton of Food Bacteria, Reading, England; DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; Chr. Hansen's, Denmark; Wiesby GmbH & Co. Germany; Kemikalia AB, Lund, Sweden; Arla R&D, Stockholm, Sweden and Medipharm AB, Kågeröd, Sweden.

Media & Growth conditions

After transferring two times in De Man-Rogosa-Sharpe (MRS) broth (pH 6.0) (De Man *et al.* 1960) (Unipath Ltd. Hampshire, England), the strains were cultured in MRS-broth spiked with different concentrations of 9,12 linoleic acid (99% pure) (Sigma Chemicals, Poole, England). The linoleic acid was added as a 5 mg ml^{-1} aqueous solution in 1% (v/v) of Tween 80 (polyoxyethylene sorbitan mono-oleate, Sigma Chemicals, Poole, England).

Each test organism was inoculated to a density of 10^6 organism ml^{-1} and incubated till stationary phase at the respective temperatures used in processing fermented dairy products, i.e. at 37°C for lactobacilli (20 hr), streptococci (25 hr); at 20°C for lactococci (72 hr) and propionibacteria (72 hr). The cultures were incubated in an anaerobic tent with an atmosphere containing 5% of carbon dioxide, 8% of hydrogen, and 87% of nitrogen.

The concentration of linoleic acid in the medium used for initial screening of CLA-producing strains was 25 μg ml^{-1}. The strains which gave positive results were, in addition, cultivated with different concentrations of free linoleic acid in MRS: 0, 10, 50, 100, 200, 500, 750, 1000 and 1500 (μg ml^{-1} of medium). The production of CLA was also tested in other growht media, i.e. sodium lactate medium (SLM, pH 7.0) (Malik *et al.* 1968) and sterilised skim-milk (pH 6.7) with the addition of 100 μg ml^{-1} linoleic acid. The sodium lactate medium contained 1.5% Trypticase® peptone (BBL Microbiology Systems, Cockeysville, MD, USA), 0,5% yeast extract (Oxoid Limited, Hampshire, England), and 1% DL-sodium lactate (98%, Sigma Chemical Co. St. Louis, Mo. USA).

Analysis

pH value: pH was measured with a Radiometer PHM83 instrument (Radiometer Analytical A/S, Bagsvaerd, Denmark).

Viable count: The viable count was determined by plating serial dilutions of suspension on MRS-agar (Oxoid Limited, Hampshire, England) for lactic acid bacteria and on sodium lactate agar for propionibacteria under anaerobic conditions as mentioned above.

Sample preparation for fatty acid analysis: Samples of the bacterial suspensions were centrifuged at room temperature at 3000 rpm (2520 x g). The bacterial pellets were washed twice with saline buffer (NaCl, 0.8%; K_2HPO_4, 0,121% and KH_2PO_4, 0.034%, pH 7.2). The supernatant and the bacteria cells were further analysed for extracellular and intracellular fatty acid composition, respectively (see further below).

Lipid extraction: Lipid extraction of the supernatant and the collected bacterial cells was based on the method described by Hara and Radin (1978). One ml sample and 2 ml isopropanol were mixed vigorously, 1.5 ml of hexane was then added and the mixture was shaken for 3 min before being centrifuged at 3000 rmp (2520 x g) for 5 min at room temperature. The upper layer was then collected. The lower layer was extracted with 1.5 ml of hexane, twice, and the supernatants were pooled with the previous hexane layer into a screw-capped test tube.

Fat Hydrolysis: The extracted lipids were hydrolysed to free fatty acid by adding 2 ml of 2M KOH in ethanol at 50°C for 30 min (Jiang *et al.* 1996).

Methylation: Methyl esters were prepared with 14% boron trifluoride in methanol in the dark (Werner *et al.* 1992).

GC Analysis: The GC analysis of methyl esters of fatty acids was based on the method described by Jiang *et al.* (1996), which was carried out with a HP5890 series II gas chromatography (Hewlett-Packard Co. Rolling Meadows, IL), fitted with a flame ionization detector and a CP Sil 88 fused silica capillary column (50 m x 0.22 mm, 0.2-mm file thickness; Chrompack, Middelburg, The Netherlands).

The temperature program was as following: 90°C held for 3 min, and then increased from 90 to 180°C at 5°C min^{-1}, where it was held for 25 min. Identification of individual fatty acids was performed by comparing the relative retention time with those of fatty acid standards. Heneicosanoic acid (Larodan Fine Chemical AB, Malmö, Sweden) was used as internal standard för GC quantification.

RESULTS

Screening for CLA producing strains

Among the 22 strains screened, three strains of *Propionibacterium freudenreichii* were found capable of producing CLA, i.e. *P. freudenreichii* subsp. *freudenreichii* ATCC 6207 (PFF), *P. freudenreichii* subsp. *freudenreichi* Propioni-6 (PFF6) and *P. freudenreichii* subsp. *sheramnii* 9093 (PFS). Further, one Bifidobacterium strain was found capable of producing CLA, i.e. *Bifidobacterium breve* ATCC 15700. Cf. Table 1.

Production of CLA by propionibacteria in MRS-broth

The fatty acid composition of the growth medium and bacterial cells of the three strains of propionibacteria capable of producing CLA was further analysed. The results showed that CLA was mainly present in the extracellular phase, i.e. growth medium, and to a much lesser extent intracellular (FIG. 1A and B). Of the possible isomers of CLA, c9,t11-/t9,c11-18:2, t10,c12-18:2, and t9,t11-/t10,t12-18:2 were formed (peak 2, 3 and 4, respectively), of which c9,t11-/t9,c11-18:2 comprised about 70 to 90% of the total CLA.

The influence of the different concentrations of linoleic acid on the formation of CLA by strains PFF, PFF6 and PFS in MRS broth is shown in FIG. 2A and B. The strains demonstrated substantial differences regarding the formation of CLA. For strain PFF, the concentration of c9,t11-/t9,c11-18:2 increased from 2.6 in the control to 17.4 $\mu g\ ml^{-1}$ of medium at a free linoleic acid level of 100 $\mu g\ ml^{-1}$ in the medium, but decreased significantly when the concentration of free linoleic acid was increased to 200 $\mu g\ ml^{-1}$. At 500 $\mu g\ ml^{-1}$ level, the growth of PFF was significantly inhibited and no CLA was formed (FIG. 2A).

For strain PFS, the formation of c9,t11-/t9,c11-18:2 was relative to the amount of free linoleic acid in the medium up to 200 $\mu g\ ml^{-1}$. Between 200 to 600

μg ml^{-1} the production of c9,t11-/t9,c11-18:2 was nearly constant, while highest value of 100.1 μg ml^{-1} was obtained at 500 μg ml^{-1} free linoleic acid level (FIG. 2A).

The highest production of c9,t11-/t9,c11-18:2 (246.4 μg ml^{-1} of medium) was formed by strain PFF6 at a concentration of free linoleic acid of 750 μg ml^{-1} of medium. When the level of free linoleic acid was increased further, the formation of c9,t11-/t9,c11-18:2 decreased but remained at a level of 101.4 μg ml^{-1}, even at a concentration of free linoleic acid as high as 1500 μg ml^{-1} (FIG. 2A).

The pattern of the formation of the trans-isomers of CLA (t9,t11-/t10,t12-18:2) were similar to that of c9,t11-/t9,c11-18:2 (FIG. 2B). However, the amounts were ten-fold less than those of c9,t11-/t9,c11-18:2, and the highest levels were obtained at much lower concentration of free linoleic acid in the medium (<200 μg ml^{-1}).

The compositions of the other major fatty acids in the media after incubation of strains PFF, PFF6 and FFS at their respective optimal levels of linoleic acid are shown in Table 2. It was found that, apart from the increase of the CLA content, no major changes in other fatty acid contents occurred for strain PFF, whereas for PFF6 and PFS, the contents of C14:0, C16:0, C16:1, and *cis*-9-C18:1 in the supernatant were significantly increased when linoleic acid was present in the medium. For PFS, an increased C18:0 content was also observed.

Formation of CLA in other media

The three CLA producing strains were also incubated in SLM media and sterilized skim milk supplemented with 100 μg ml^{-1} of linoleic acid to investigate any effects of the media on CLA formation (Table 3). When strains were grown in SLM medium, only strain PFF6 was capable of converting about 50% of free linoleic acid into CLA, whereas no CLA was formed by the other two strains. In skim-milk, on the other hand, all three strains were capable of producing CLA and about 60 to 90% of the free linoleic acid was converted to CLA (Table 3).

Inhibitory effect of free linoleic acid on the growth of propionibacteria

The effects of free linoleic acid (25 μg ml^{-1}) on the growth of the screened bacteria in MRS-broth is shown in Table 1. Most of the strains were clearly inhibited. Regarding the strains of propionibacteria, those able to produce CLA

were more susceptible than those not producing CLA, i.e. the growths of *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* were clearly inhibited whereas *P. jensenii* and *P. thoenii* were not affected.

The effects of the different concentrations of free linoleic acid on the growth of the three CLA producing strains were also studied in MRS medium by determining the final pH and the viable count. The results (FIG. 3A) show a negative correlation between the total bacteria count and the concentration of linoleic acid in MRS medium, and the order of the susceptibility to linoleic acid was PFF > PFS > PFF6, which was in agreement with the final pH drop after incubation (FIG. 3B), where the growth of PFF6 was least affected by addition of free linoleic acid into the medium.

The effects of free linoleic acid (100 μg ml^{-1}) on the growth of the CLA producing strains in SLM and sterilized skim-milk are shown in Table 3. The growths of all three strains, expressed as viable counts and pH, were significantly inhibited by the presence of linoleic acid in SLM. In skim-milk, only the growth of PFF6 was significantly inhibited by linoleic acid but the inhibitory effects were less than in SLM. The final pH of PFF and PFS strains in SLM were in contrast to that in MRS medium: lower values of pH were obtained in the presence of linoleic acid than in the controls. In skim-milk, the effect of linoleic acid on the final pH values was much less than in the SLM.

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In the experimental support on which the present invention is based, 22 strains of commonly used dairy starter cultures were screened for their ability to produce CLA *in vitro*. Three strains of *Propionibacterium freudenreichii* subsp. *freudenreichii* and *shermani* and one strain of *Bifidobacterium breve* were found capable of producing CLA from free linoleic acid. Propionibacteria are Gram-positive, short rods that grow under anaerobic conditions by using glucose or lactate as energy sources and produce propionic and acetic acid (Hettinga and Reinbold, 1972). These organisms are important in several industrial applications, e.g. they are essential in development of the characteristic flavour and eye formation in Swiss-type cheeses. Bifidobacteria are Gram-positive, rods of various shapes which grow under anaerobic conditions. They are saccharoclastic, and acetic and lactic acids are the main products (Cummins and Johnson, 1986). These microorganisms are widely used in dairy industry to produce fermented milk

products regarded as healthy food.

The CLA formed by the food grade bacteria was mainly found in the extracellular phase and of the different CLA isomers, c9,t11-/t9,c11-18:2 was found to represent 70-90% of total CLA formed. This is similar to the proportion found in milk and dairy products (Chin *et al.* 1992).

The antibacterial effect of free linoleic acid has been known for many years and growth of many of the strains screened according to this invention were inhibited to certain extent upon inclusion of 25 μg ml^{-1} linoleic acid into the growth media. It was also observed that the effects of free linoleic acid on the growth of the tested propionibacteria were different, i.e. *P. freudenreichii* subsp. *freudenreichii* and subsp. *shermanii*, were inhibited whereas *P. jensenii* and *P. thoenii* were not inhibited. Similar result has been reported by Boyaval *et al.* (1995), that free linoleic acid has a strong negative effect on growth and metabolism of *P. freudenreichii* subsp. *shermanii*. Furthermore, Moss *et al.* (1969) classified the propionibacteria into two groups based on their differences in the fatty acid composition, i.e. *P. freudenreichii* subsp. *freudenreichii* and subsp. *shermanii* on one hand and, on the other hand, *P. jensenii* and *P. thoenii*. This grouping is thus in agreement with the different susceptibility of propionibacteria to linoleic acid found according to the present invention. It is also interesting to note that those strains of propionibacteria which were able to produce CLA were inhibited by free linoleic acid and vice versa. Among the three CLA-producing strains, a positive correlation between CLA production and their ability of tolerance to free linoleic acid was observed, which suggested that conversion of free linoleic acid to CLA might function as a detoxification mechanism.

The free linoleic acid in the medium was converted mainly to c9,t11-/t9,c11-18:2. The increase of cis-9-C18:1 in the extracellular phase indicates that some of the c9,t11-/t9,c11-18:2 was further hydrogenated to cis-9-C18:1. This is different from the pathway of biohydrogenation of linoleic acid by *B. fibrisolvens* in the bovine rumen in which c9,t11-18.2 is hydrogenated to trans-11-C18:1.

The formation of CLA was affected by the different media used. The production of CLA was less in SLM medium and the inhibitory effects of linoleic acid on the bacteria were stronger than in MRS and in skim-milk. The reason for this is still unclear but one likely explanation is the factors known to neutralize the inhibitory effects of fatty acids such as linoleic acid. For instance, MRS-broth contained 0.1% of Tween 80, while skim-milk contained 3% of milk protein. The growth stimulation property of Tween 80 and its properties of neutralization of

the antimicrobial effect of fatty acids have been well demonstrated in the past (Baker *et al.* 1983; Cummins and Johnson 1986; Dubos 1947; Ledeoma *et al.* 1977). Furthermore, the presence of proteins can also counteract the inhibition of fatty acids (Boyaval *et al.* 1995; Dubos 1947). The results observed according to the invention thus concur with the opinion that the presence of certain surface active substances, such as Tween 80 or proteins, plays a important role in the recovery of inhibitory effects of free linoleic acid on the growth of propionibacteria and hence the production of CLA.

The surprising discovery that strains of propionibacteria and bifidobacteria used as starter cultures in the dairy industry are capable of converting free linoleic acid to CLA opens up interesting perspectives for producing fermented food products and particularly dairy products enriched in CLA.

The application of using certain food grade bacteria, in particular certain dairy starter cultures, to produce food or feed products having enhanced CLA levels is exemplified below by the following Examples wherein fermented milk products enriched in CLA are produced. Throughout the Examples, *Propionibacterium freudenreichii* subsp. *shermanii* 9093 and *Propionibacterium freudenreichii* subsp. *freudenreichii* Propioni-6 were used as propionic bacteria strains.

EXAMPLE 1

Different levels of free linoleic acid (0, 100, 300, 500, 1000, 1500, and 2000 μg/ml) was added into milk (fat content 3%, homogenized) and propionic bacteria were incubated in this milk at 22°C for 72 hours. The highest level of CLA produced was 20 mg/g of fat, in the samples that contained 1000 μg free linoleic acid per ml of milk. Therefore, the optimum level of free linoleic acid needed for CLA production in milk seems to be about 1000 μg/ml.

EXAMPLE 2

Milk of 3% fat supplemented with 1000 mg/ml free linoleic acid was incubated with propionic bacteria, samples were collected every 24 hours. The highest level of CLA (20 mg/g of fat) was found after 24 hours incubation.

EXAMPLE 3

Milk of 3% fat content, supplemented with 1000 mg/ml of free linoleic acid, was incubated with propionic bacteria for 24 hours, at ambient temperature; afterwards, freshly prepared starter culture (1%, LD-culture) was added and incubated for additional 20 hours. The milk was coagulated and the final pH was 4.6. The highest level of CLA was about 23 mg/g fat (4.5 mg/g in control samples). The texture of this fermented milk was similar to that of fermented control milk, and the taste was well accepted.

EXAMPLE 4

Skim milk and cream having a fat content of 12% were supplemented with 1000 mg/ml of free linoleic acid and incubated with propionic bacteria for 24 hours. The highest level of CLA in the skim milk was about 200 mg of CLA per g fat in the skim milk and about 10 mg/g fat in the cream.

EXAMPLE 5

The fermented milk product of Examples 3 and 4 was concentrated by heating and centrifugation in a quarg separator. Most of the CLA was retained in the cheese formed and only a minor amount was lost with the whey. Quarg products of different fat content but with good flavour and enriched in CLA were obtained.

EXAMPLE 6

Example 3 was repeated but the propionic acid bacteria and the starter culture were added at the same time. The amount of starter culture was reduced to 5% of the amount normally used and the temperature was reduced. By proceeding in this way, the fermentation was completed in 26 hours and the same amount of CLA as in Example 3 was obtained.

EXAMPLE 7

Milk of 3% fat content, supplemented with 1000 mg/ml of free linoleic acid,

was incubated with *Bifidobacterium breve* ATCC 15700 for 18 hours at 37°C; afterwards freshly prepared yoghurt culture was added as a starter and the milk incubated for additional 6 hours. The milk was coagulated and the final pH was 4.5. The highest level of CLA was about 28 mg/g fat (7.5 mg/g in control samples). The texture of this fermented milk was similar to that of fermented control milk, and the taste was well accepted.

****

As is evident from the Examples above, it is possible to produce fermented dairy products with an enriched CLA content of at least 15 mg/100 g.

As indicated under "BACKGROUND OF THE INVENTION", the level of CLA in raw milk can be increased by feeding the cows with a suitable feeding regimen. Thus, it is possible to reach by suitable feeding, a level of 20 mg of CLA per gram of fat. Since the CLA-enriched propionibacterium-fermented and bifidobacterium-fermented milk acording to the invention also could contain 20 mg of CLA/g of fat, thus, in total, the CLA level can be increased about 10 times (about 40 mg of CLA/g of fat) which makes it possible for the consumer to obtain the required amount of CLA from dairy products.

Table 1. Screening of strains for their capacity of producing CLA from free linoleic acid (25µg/ml) in MRS broth. For incubation conditions, see Materials & Methods.

Strains	Source[1]	Inhibitory on growth[2]	Formation of CLA[3]
Lactobacillus acidophilus ATCC4356	A	+	0
Lactobacillus bulgaricus	C	+	0
Lactobacillus casei	D	-	0
Lactobacillus casei F-19	G	-	0
Lactobacillus fermentum	D	+	0
Lactobacillus helveticus ATCC 15009	B	-	0
Lactobacillus reuteri ATCC 23272	B	+	0
Lactococcus lactis subsp. *lactis* NCFB 176	A	+	0
Lactococcus lactis subsp. *lactis* ATCC 19435	A	+	0
Lactococcus lactis subsp. *cremoris* ATCC 19257	A	+	0
Lactococcus lactis subsp. *cremoris* NCFB 924	A	+	0
Streptococcus salivarius subsp. *thermophilus*	C	+	0
Streptococcus salivarius subsp. *thermophilus* ATCC 19258	A	+	0
Bifidobacterium bifidum ATCC 29521	A	+	0
Bifidobacterium breve ATCC 15700	A	+	*
Bifidobacterium longum	H	+	0
Propionibacterium freudenreichii subsp. *freudenreichii* ATCC 6207	B	+	*
Propionibacterium freudenreichii subsp. *freudenreichii* Propioni-6	C	+	*
Propionibacterium freudenreichii subsp. *shermanii* PS-1	F	+	0
Propionibacterium freudenreichii subsp. *shermanii* 9093	E	+	*
Propionibacterium jensenii ATCC 4867	B	-	0
Propionibacterium thoenii ATCC 4874	B	-	0

[1] Source: A) National Collection of Food Bacteria, Agricultural and Food Research Council, Institute of Food Research, Reading, England; B) DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Braunschweig, Germany; C) Wiesby GmbH & Co. KG, Niebüll, Germany; D) Arla FoU. Stockholm, Sweden; E) Kemikalia AB, Lund, Sweden; F) CHR. Hansen's, Copenhagen, Denmark; G) Mediharm AB. Kågeröd, Sweden; H) Morinaga Milk Industry Co. Ltd. Tokyo, Japan.

[2] +: growth was inhibited for more than 50%
-: growth was not clearly inhibited

[3] 0: CLA not produced
*: CLA produced

Table 2. Mean values (n = 3) of the fatty acid composition of the medium after growth of *Propionibacterium freudenreichii* subsp. *freudenreichii* ATCC 6207 (PFF); *Propionibacterium freudenreichii* subsp. *freudenreichii* Propioni-6 Wiesby (PFF6); or *Propionibacterium freudenreichii* subsp. *shermanii* 9093, Kemikalia (PFS), in MRS broth. Incubation under anaerobic conditions at 20°C for 72 h

	Linoleic acid*	C14:0	C16:0	C16:1	C18:0	cis9-18:1	trans11-18:1	C18:2	c/tc-9,11-CLA	Total CLA
						μg ml^{-1} of medium				
MRS-broth†	0	2.5	7.3	3.8	4.1	44.7	2.2	0.1	1.2	1.2
Strain										
PFF	0	2.1	6.1	4.5	4.7	72.1	2.5	0.3^{a}	2.6^{a}	6.4^{a}
	100	2.0	6.1	4.5	4.1	72.3	2.7	47.5^{b}	17.4^{b}	23.2^{b}
PFF6	0	1.4^{a}	4.6^{a}	3.7^{a}	4.7	65.1^{a}	2.9	0.1^{a}	2.6^{a}	5.6^{a}
	750	5.0^{b}	23.1^{b}	10.9^{b}	3.8	103.6^{b}	2.3	380.3^{b}	246.4^{b}	265.3^{b}
PFS	0	1.2^{a}	3.9^{a}	6.6^{a}	4.5^{a}	93.9^{a}	4.8	3.5^{a}	3.1^{a}	9.1^{a}
	500	3.6^{b}	13.5^{b}	10.4^{b}	7.8^{b}	130.8^{b}	4.7	289.5^{b}	100.5^{b}	111.8^{b}

*Concentration of linoleic acid in the medium which resulted in highest CLA production.

†Medium without the growth of bacteria

a,b Within each comparable pair, means marked with different superscripts are significantly different ($P < 0.01$).

Table 3. Means (n = 3) of the growth and production of CLA (μg ml^{-1}) by *Propionibacterium freudenreichii* subsp. *freudenreichii* ATCC 6207 (PFF); *Propionibacterium freudenreichii* subsp. *freudenreichii* Propioni-6, Wiesby (PFF6); and *Propionibacterium freudenreichii* subsp. *shermanii* 9093, Kemikalia (PFS), in sodium lactate broth and sterilized skim-milk. Incubation under anaerobic conditions at 20°C for 72 h

		Final pH		Final VC*		ct/tc-9,11-CLA		Total CLA	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Sodium lactate medium									
PFF	Control	6.81[a]	0.01	131[a]	5.21	0.1	0.0	0.0	0.0
	LA†	6.72[b]	0.01	<1[b]	0.32	0.1	0.0	0.0	0.0
PFF6	Control	6.52[a]	0.01	124[a]	3.72	1.2[a]	0.0	1.2[a]	0.1
	LA	6.93[b]	0.01	11[b]	1.25	35.9[b]	1.2	42.0[b]	1.6
PFS	Control	6.70[a]	0.01	240[a]	9.21	3.1	0.1	3.1	0.1
	LA	6.20[b]	0.00	10[b]	1.01	3.6	0.2	3.6	0.1
Skim-milk									
PFF	Control	6.26	0.01	66	1.27	2.1[a]	0.2	2.1[a]	0.2
	LA	6.25	0.01	59	2.16	54.6[b]	2.3	62.5[b]	2.6
PFF6	Control	5.37[a]	0.01	228[a]	4.21	2.1[a]	0.1	2.1[a]	0.1
	LA	5.49[b]	0.01	155[b]	4.11	77.0[b]	3.3	92.3[b]	2.7
PFS	Control	6.07	0.01	30	1.21	2.7[a]	0.3	2.7[a]	0.3
	LA	6.05	0.02	32	1.17	73.0[b]	6.6	84.5[b]	9.7

* Viable count x 10^{-7} cfu ml^{-1} of medium.

† Linoleic acid 100 μg ml^{-1} of medium.

[a,b] Within each comparable pair, means marked with different superscripts are significantly different ($P < 0.01$).

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CLAIMS

1. Use of selected bacterial strains for producing conjugated linoleic acid (CLA), said strains being found among food grade bacteria and having the ability of forming CLA *in vitro* by converting linoleic acid to CLA.

2. Use according to claim 1, characterized in that said strains produce CLA *in vitro*, the major isomer of which is cis-9,trans-11-octadecadienoic acid (c9,t11-18:2).

3. Use according to claim 1 or 2, characterized in that said strains are selected from dairy starter cultures.

4. Use according to claim 3, characterized in that said starter cultures are selected from strains of propionibacteria and bifidobacteria.

5. Use according to claim 4, characterized in that said strains are selected from *Propionibacterium freudenreichii (P.f.)* subsp. *freudenreichii*, *P.f.* subsp. *sheramnii* and *Bifidobacterium breve*.

6. Use according to claim 5, characterized in that said strains are *P.f.* subsp. *freudenreichii* ATCC 6207, *P.f.* subsp. *freudenreichii* Propioni-6, *P.f.* subsp. *sheramnii* 9093 and *Bifidobacterium breve* ATCC 15700.

7. Use according to any of claims 1-6 for producing food or feed products enriched in CLA.

8. Use according to claim 7, characterized in that said food product is a dairy product or an imitation dairy product.

9. Use according to any of claims 1-6 for producing a pharmaceutical product containing CLA as active ingredient.

10. Food or feed product enriched in CLA by using CLA-producing strains as defined in any of claims 1-6.

11. Product according to claim 10, characterized in being a dairy product or an imitation dairy product.

12. Product according to claim 11, characterized by being a milk-based product.

13. Product according to claim 12, characterized by being fermented milk, cheese, processed cheese, yellow fat, dairy spread or ice-cream.

14. Product according to any of claims 11-13, characterized by having an enriched level of CLA of at least 10 mg/100 g product, preferably at least 15 mg/100 g product.

15. Pharmaceutical product containing CLA as active ingredient.

16. Process for the preparation of a CLA-enriched food or feed product according to any of claims 10-14, characterized by fermenting or incubating the product to be enriched in CLA using the CLA-producing bacterial strains as defined in any of claims 1-6, under CLA-producing conditions in the presence of linoleic acid.

17. Process according to claim 16 for the preparation of CLA-enriched milk, characterized by fermenting or incubating the milk to be CLA-enriched with a propionibacterium or bifidobacterium strain as defined in claim 5 or 6, in the presence of linoleic acid for up to 72 hours.

18. Process according to claim 17, characterized by fermenting or incubating the milk to be CLA-enriched at about 22°C for about 24 hours at a linoleic acid concentration of up to about 1 g per liter of milk.

19. Process according to claim 16, characterized by conducting the fermentation in the presence of an emulgator.

20. Process according to claim 19, characterized in that the emulgator is Tween 80 or lecithins.

21. Additive for the preparation of products according to claim 10 or 15, characterized by being CLA as such or CLA in concentrated form, prepared by fermen-

tation of a linoleic acid-containing substrate using a CLA-producing bacterial strain as defined in any of claims 1-6.

22. Process for the preparation of the additive of claim 21, characterized by fermenting, under conditions promoting the production of CLA, a substrate containing linoleic acid with a CLA-producing bacterial strain, as defined in any of claims 1-6, and recovering the CLA as such or in concentrated form.

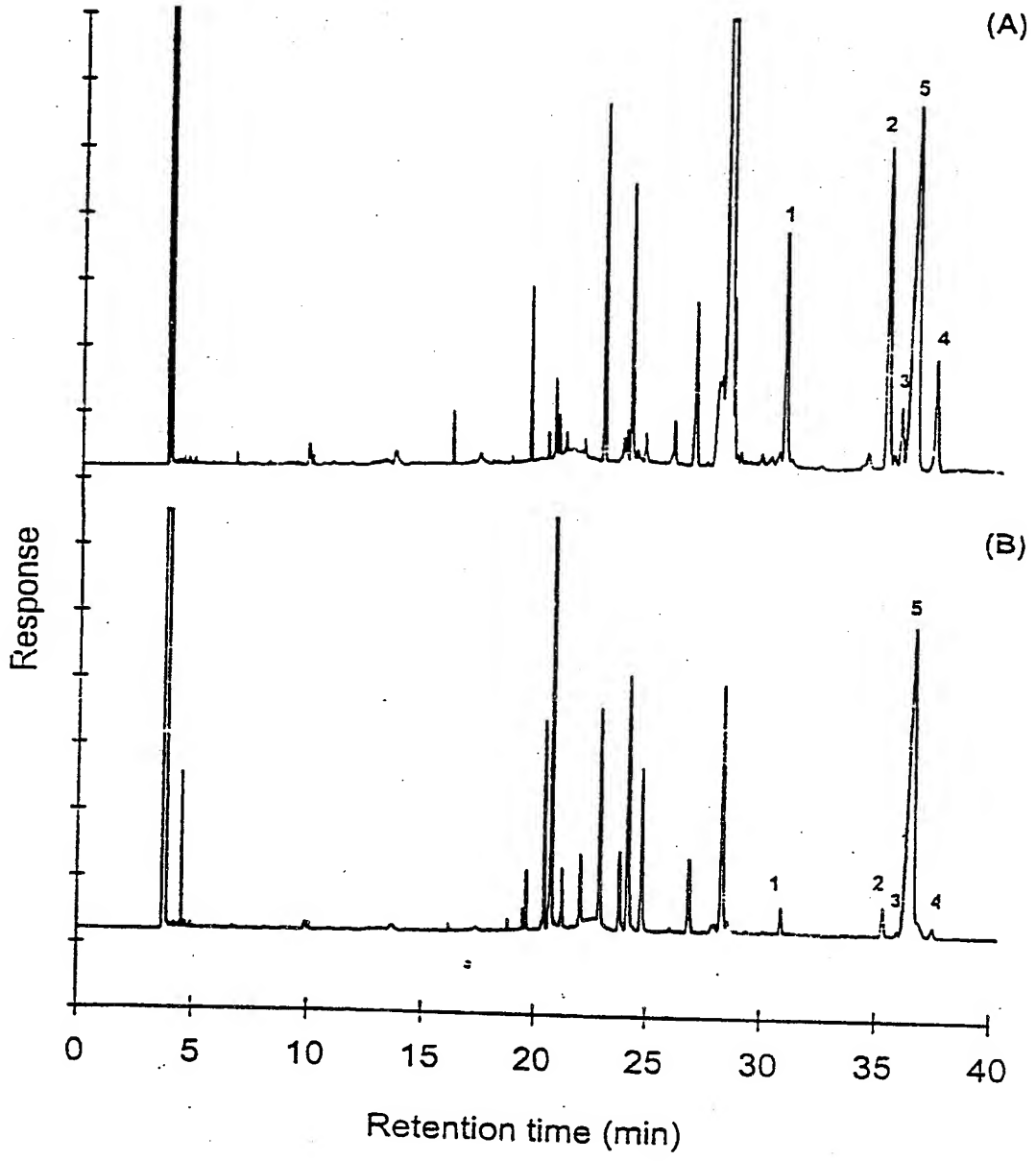

FIG. 1A and B

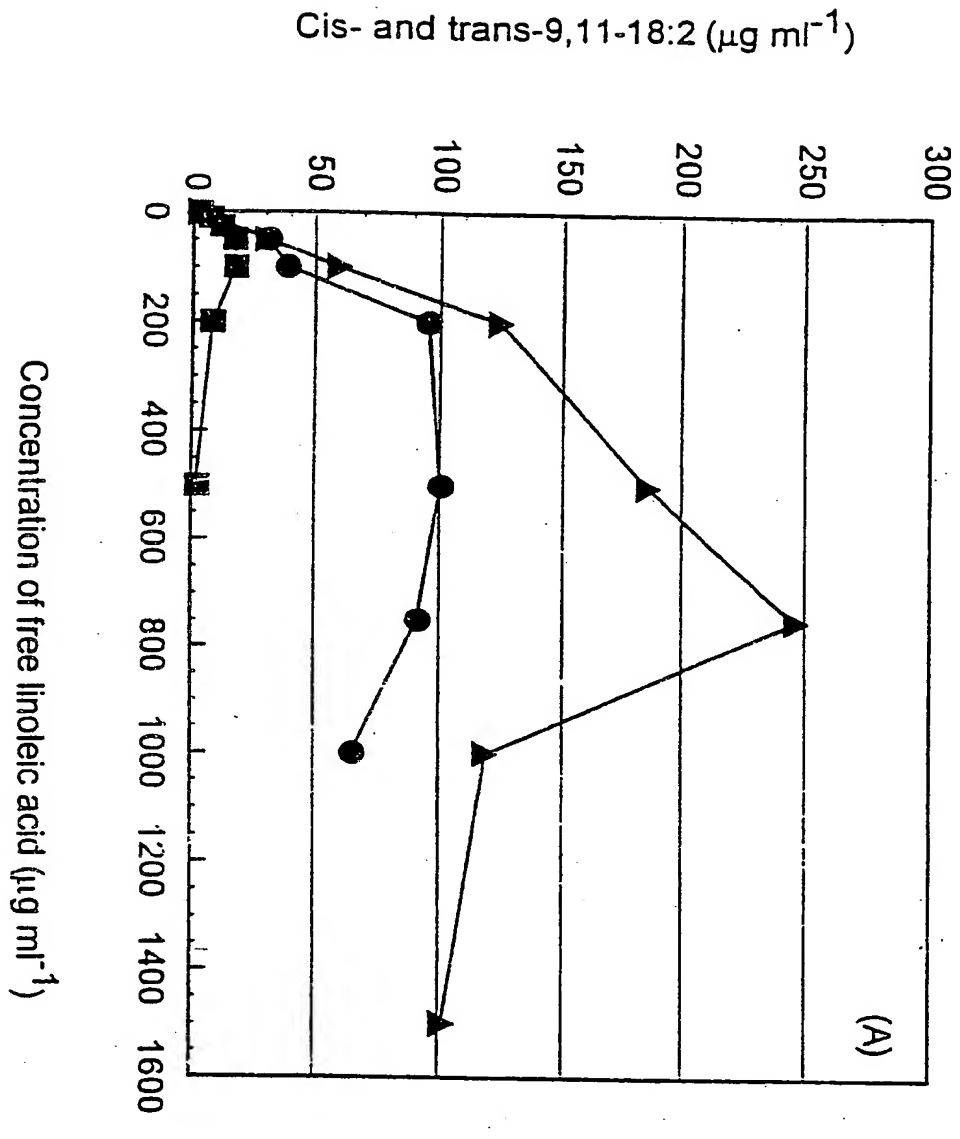
Cis- and trans-9,11-18:2 (µg ml-1)
0
50
100
150
200
250
300
0
200
400
600
800
1000
1200
1400
1600
Concentration of free linoleic acid (µg ml-1)
(A)

FIG. 2A

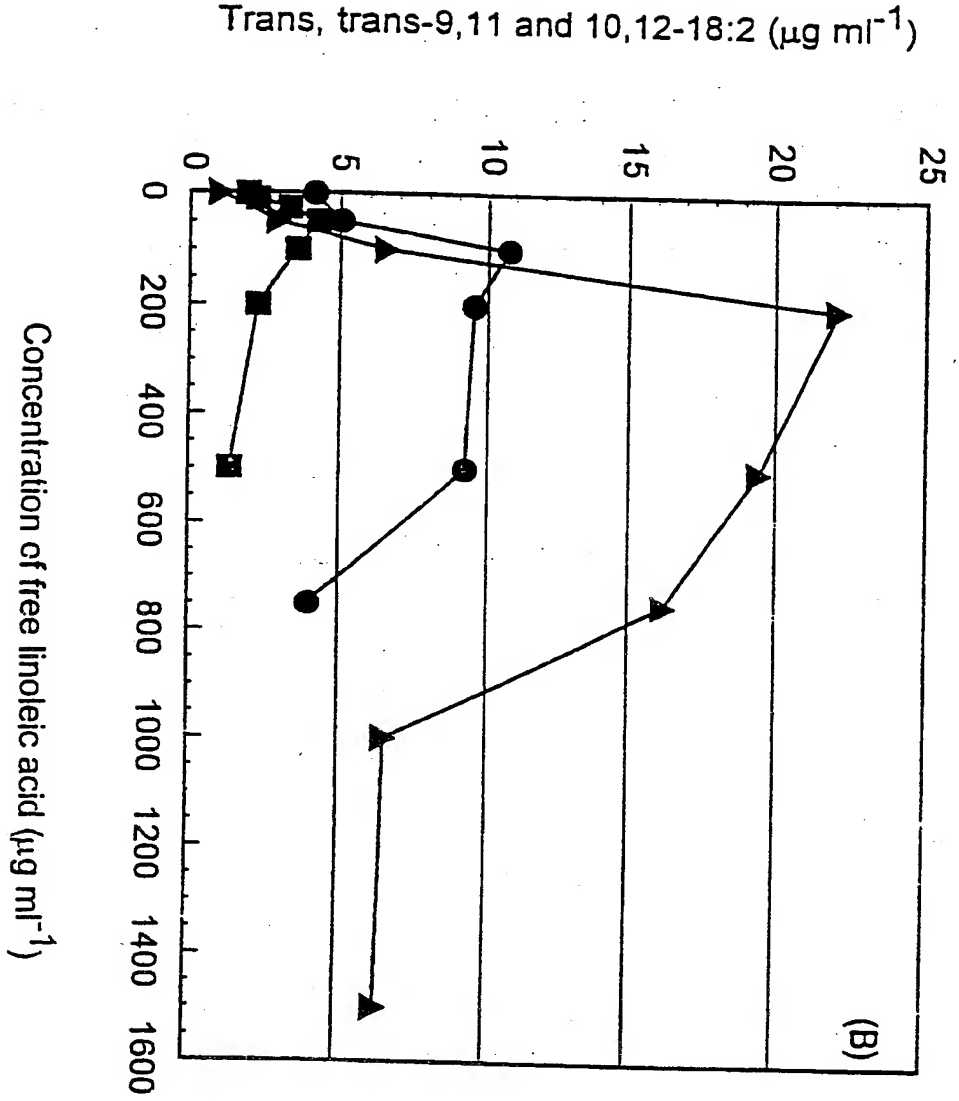
Trans, trans-9,11 and 10,12-18:2 (µg ml-1)
0
5
10
15
20
25
0
200
400
600
800
1000
1200
1400
1600
Concentration of free linoleic acid (µg ml-1)
(B)

FIG. 2B

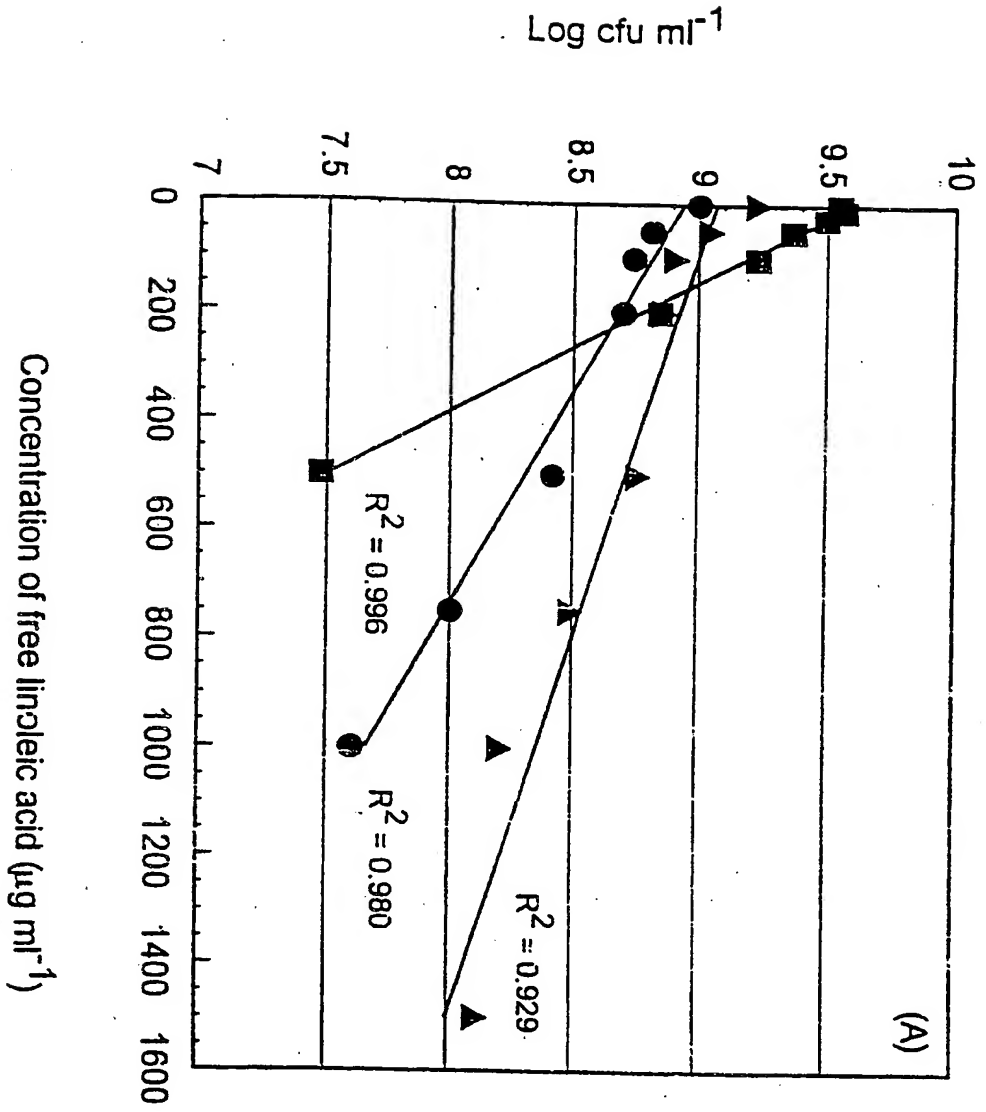
Log cfu ml-1
7
7.5
8
8.5
9
9.5
10
0
200
400
600
800
1000
1200
1400
1600
Concentration of free linoleic acid (µg ml-1)
R2 = 0.996
R2 = 0.980
R2 = 0.929
(A)

FIG. 3A

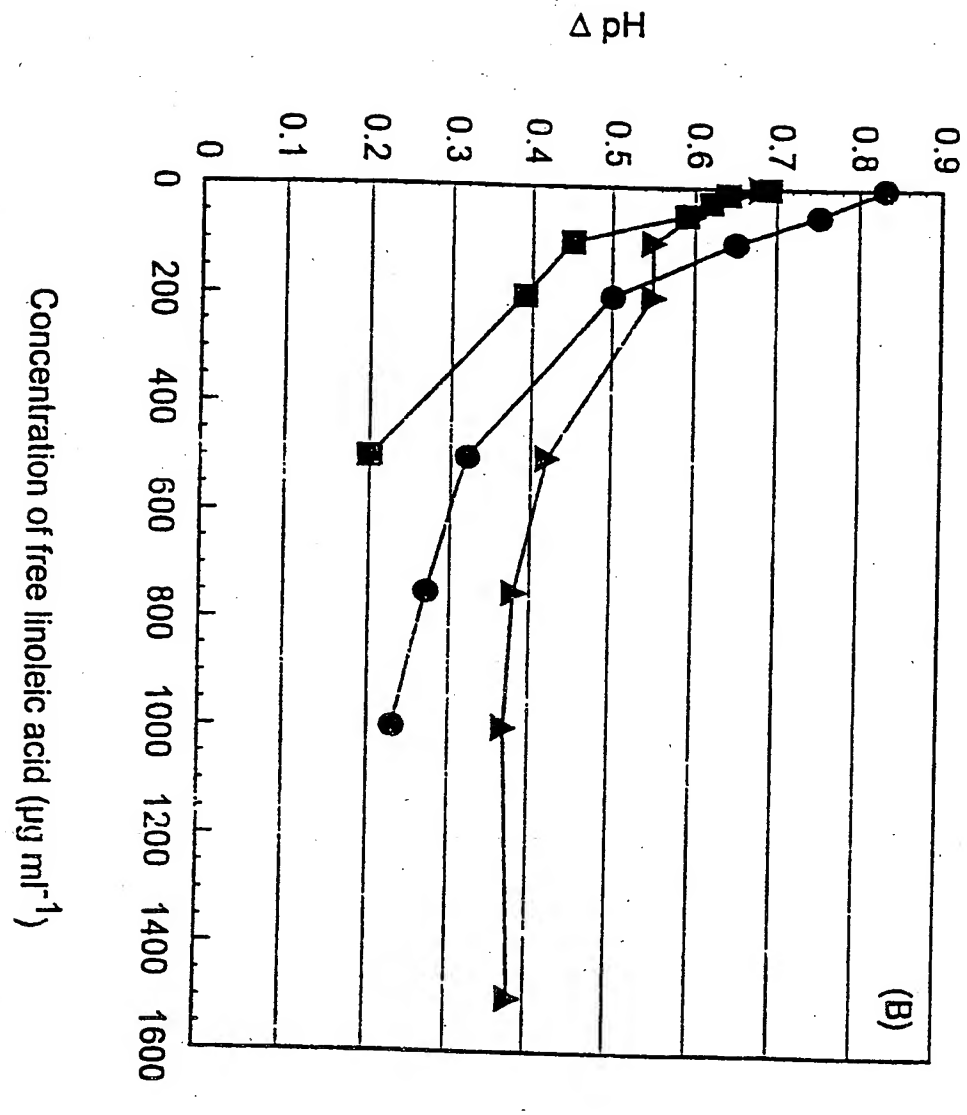
Δ pH
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
200
400
600
800
1000
1200
1400
1600
Concentration of free linoleic acid (μg ml-1)
(B)

FIG. 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/02207

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 7/64, A23C 9/12, A61K 31/20, A23K 1/16
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P, A23C, A61K, A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, MEDLINE, BIOSIS, DBA, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9638137 A1 (WISCONSIN ALUMNI RESEARCH FOUNDATION), 5 December 1996 (05.12.96), especially claims 5 and 6 --	1-22
P,X	Dialog Information Services, File 155, Medline, Dialog accession no. 09634298, Medline accession no. 98388632, Jiang J. et al: "Production of conjugated linoleic acid by dairy starter cultures", J Appl Microbiol ((ENGLAND) Jul 1998, 85(1) p95-102 --	1-22

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1999	19 -03- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/02/99

International application No.
PCT/SE 98/02207

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9638137 A1	05/12/96	AU 5253596 A	18/12/96
		EP 0831804 A	01/04/98
		US 5674901 A	07/10/97
		US 5827885 A	27/10/98
		US 5856149 A	05/01/99